REFERENCES AND NOTES

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3. D. Shalon, thesis, Stanford University (1995); ——— and P. O. Brown, in preparation. Microarrays were fabricated on poly-L-lysine–coated microscope slides (Sigma) with a custom-built arraying machine fitted with one printing tip. The tip loaded 1 μl of PCR product (0.5 mg/ml) from 96-well microtiter plates and deposited ~0.005 μl per slide on 40 slides at a spacing of 500 μm. The printed slides were rehydrated for 2 hours in a humid chamber, snap-dried at 100°C for 1 min, rinsed in 0.1% SDS, and treated with 0.05% succinic anhydride prepared in buffer consisting of 50% 1-methyl-2-pyrrolidinone and 50% boric acid. The cDNA on the slides was denatured in distilled water for 2 min at 90°C immediately before use. Microarrays were scanned with a laser fluorescent scanner that contained a computer-controlled XY stage and a microscope objective. A mixed gas, multiline laser allowed sequential excitation of the two fluorophores. Emitted light was split according to wavelength and detected with two photomultiplier tubes. Signals were read into a PC with the use of a 12-bit analog-to-digital board. Additional details of microarray fabrication and use may be obtained by means of e-mail (pbrown@cmgm.stanford.edu).
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5. Polyadenylated [poly(A)$^+$] mRNA was prepared from total RNA with the use of Oligotex-dT resin (Qiagen). Reverse transcription (RT) reactions were carried out with a StrataScript RT-PCR kit (Stratagene) modified as follows: 50-μl reactions contained 0.1 μg/μl of *Arabidopsis* mRNA, 0.1 ng/μl of human AChR mRNA, 0.05 μg/μl of oligo(dT) (21-mer), 1× first strand buffer, 0.03 U/μl of ribonuclease block, 500 μM deoxyadenosine triphosphate (dATP), 500 μM deoxyguanosine triphosphate, 500 μM dTTP, 40 μM deoxycytosine triphosphate (dCTP), 40 μM fluorescein-12-dCTP (or lissamine-5-dCTP), and 0.03 U/μl of StrataScript reverse transcriptase. Reactions were incubated for 60 min at 37°C, precipitated with ethanol, and resuspended in 10 μl of TE (10 mM tris-HCl and 1 mM EDTA, pH 8.0). Samples were then heated for 3 min at 94°C and chilled on ice. The RNA was degraded by adding 0.25 μl of 10 N NaOH followed by a 10-min incubation at 37°C. The samples were neutralized by addition of 2.5 μl of 1 M tris-Cl (pH 8.0) and 0.25 μl of 10 N HCl and precipitated with ethanol. Pellets were washed with 70% ethanol, dried to completion in a speedvac, resuspended in 10 μl of H_2O, and reduced to 3.0 μl in a speedvac. Fluorescent nucleotide analogs were obtained from New England Nuclear (DuPont).
6. Hybridization reactions contained 1.0 μl of fluorescent cDNA synthesis product (5) and 1.0 μl of hybridization buffer [10× saline sodium citrate (SSC) and 0.2% SDS]. The 2.0-μl probe mixtures were aliquoted onto the microarray surface and covered with cover slips (12 mm round). Arrays were transferred to a hybridization chamber (3) and incubated for 18 hours at 65°C. Arrays were washed for 5 min at room temperature (25°C) in low-stringency wash buffer (1× SSC and 0.1% SDS), then for 10 min at room temperature in high-stringency wash buffer (0.1× SSC and 0.1% SDS). Arrays were scanned in 0.1× SSC with the use of a fluorescence laser-scanning device (3).
7. Samples of poly(A)$^+$ mRNA (4, 5) were spotted onto nylon membranes (Nytran) and crosslinked with ultraviolet light with the use of a Stratalinker 1800 (Stratagene). Probes were prepared by random priming with the use of a Prime-It II kit (Stratagene) in the presence of [^{32}P]dATP. Hybridizations were carried out according to the instructions of the manufacturer. Quantitation was performed on a PhosphorImager (Molecular Dynamics).
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12. The laser fluorescent scanner was designed and fabricated in collaboration with S. Smith of Stanford University. Scanner and analysis software was developed by R. X. Xia. The succinic anhydride reaction was suggested by J. Mulligan and J. Van Ness of Darwin Molecular Corporation. Thanks to S. Theologis, C. Somerville, K. Yamamoto, and members of the laboratories of R.W.D. and P.O.B. for critical comments. Supported by the Howard Hughes Medical Institute and by grants from NIH (R21HG00450) (P.O.B.) and R37AG00198 (R.W.D.)] and from NSF (MCB9106011) (R.W.D.) and by an NSF graduate fellowship (D.S.). P.O.B. is an assistant investigator of the Howard Hughes Medical Institute.

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Gene Therapy in Peripheral Blood Lymphocytes and Bone Marrow for ADA$^-$ Immunodeficient Patients

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Adenosine deaminase (ADA) deficiency results in severe combined immunodeficiency, the first genetic disorder treated by gene therapy. Two different retroviral vectors were used to transfer ex vivo the human ADA minigene into bone marrow cells and peripheral blood lymphocytes from two patients undergoing exogenous enzyme replacement therapy. After 2 years of treatment, long-term survival of T and B lymphocytes, marrow cells, and granulocytes expressing the transferred ADA gene was demonstrated and resulted in normalization of the immune repertoire and restoration of cellular and humoral immunity. After discontinuation of treatment, T lymphocytes, derived from transduced peripheral blood lymphocytes, were progressively replaced by marrow-derived T cells in both patients. These results indicate successful gene transfer into long-lasting progenitor cells, producing a functional multilineage progeny.

Severe combined immunodeficiency associated with inherited deficiency of ADA (*1*) is usually fatal unless affected children are kept in protective isolation or the immune system is reconstituted by bone marrow transplantation from a human leukocyte antigen (HLA)–identical sibling donor (*2*). This is the therapy of choice, although it is available only for a minority of patients. In recent years, other forms of therapy have been developed, including transplants from haploidentical donors (*3*, *4*), exogenous enzyme replacement (*5*), and somatic-cell gene therapy (*6*–*9*).

We previously reported a preclinical model in which ADA gene transfer and expression successfully restored immune functions in human ADA-deficient (ADA$^-$) peripheral blood lymphocytes (PBLs) in immunodeficient mice in vivo (*10*, *11*). On the basis of these preclinical results, the clinical application of gene therapy for the treatment of ADA$^-$ SCID (severe combined immunodeficiency disease) patients who previously failed exogenous enzyme replacement therapy was approved by our Institutional Ethical Committees and by the Italian National Committee for Bioethics (*12*). In addition to evaluating the safety and efficacy of the gene therapy procedure, the aim of the study was to define the relative role of PBLs and hematopoietic stem cells in the long-term reconstitution of immune functions after retroviral vector–mediated ADA gene transfer. For this purpose, two structurally identical vectors expressing the human ADA complementary DNA (cDNA), distinguishable by the presence of alternative restriction sites in a nonfunctional region of the viral long-terminal repeat (LTR), were used to transduce PBLs and bone marrow (BM) cells independently. This procedure allowed identification of the origin of

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cells, and their progeny, after gene transfer.

This combined therapy and marking strategy allowed us to investigate directly in humans some of the basic questions related to the potential of retroviral vectors for gene therapy in cells of the hemato-lymphopoietic lineages. Although gene transfer into human hematopoietic progenitors (13, 14), peripheral blood stem cells (15), and PBLs (16–18) has been extensively demonstrated in vitro, the potential for long-term survival in vivo after the manipulations required for retroviral vector gene transfer remains to be proven. In addition, this study allowed us to study the feasibility of gene transfer into hematopoietic stem and progenitor cells, and the potential for long-term persistence of differentiated cells in a context different from high-dose chemotherapy and BM transplantation (19–21). In this system, however, the positive selection may represent an absolute requirement for favoring the appearance of vector-transduced cells.

In ADA patients, failure of the immune system to develop is due to the sensitivity of lymphocytes or their precursors to the toxic effects of accumulated ADA substrates (22). Because it is possible to reduce the levels of toxic metabolites in ADA cells by providing exogenous ADA (23), a nonselective form of ADA replacement (that is, transfusion of irradiated red cells from normal individuals) has been used to treat ADA patients (24). An improved form of treatment was developed by covalent attachment of polyethylene glycol (PEG) to the purified bovine enzyme (23, 25). PEGilation appears to block access of degradative enzymes, antibodies, and antigen-presenting cells to the protein surface, thereby inhibiting clearance from the circulation (26–28) and prolonging ADA plasma half-life from a few minutes to 24 hours (23). The main biochemical consequences of ADA deficiency are almost completely reversed by PEG-ADA treatment (23), resulting in an increase in circulating T lymphocytes and improvement of cellular immune functions (23, 29).

In our study, treatment in two patients [G.B., patient 1; A.R., patient 2 (30); both about 2 years of age] was initiated with weekly intramuscular injections of increasing doses of PEG-ADA (20 and 30 U per kilogram of body weight) until plasma ADA activity could be maintained at least in the normal range of total blood activity. The range of ADA activity was stable between 20 and 40 μmol hour^{-1} ml^{-1}. Before initiation of treatment, both patients had nearly undetectable intracellular ADA activity, and lymphopenia was observed in both patients. Approximately 50% of blood mononuclear cells reacted with monoclonal antibodies to T cell surface antigens, and their proliferative response to mitogens ranged from virtually undetectable to 10% of normal controls. Both patients showed some response in mixed lymphocyte culture, although they produced no specific antibody and showed no antigen-restricted T cell response. Residual immune functions were probably due to previous irradiated red cell transfusions. During the first year of PEG-ADA treatment, lymphocyte counts and proliferative responses to phytohemagglutinin (PHA) normalized.

Immunological reconstitution resulted in increased isohemoagglutinin titer and in cellular and antibody responses to vaccination with tetanus toxoid (TT) (31). Associated with reconstitution of immune functions was the complete reversion of all clinical signs of immunodeficiency. However, as reported elsewhere (29), the initial reconstitution in this case was limited by the failure to maintain PBL counts and, more markedly, antigen-specific and nonspecific proliferative responses. At that point, the two patients met the conditions that define PEG-ADA treatment failure, as reported in our approved clinical protocol (12). Failure of treatment was defined by an extensive number of laboratory parameters and immunological assays (12). In both patients, failure of treatment was observed in the absence of any acute illness or open infection episodes and was confirmed in three separate determinations. Waiting for potential recurrence of clinical symptoms such as infectious episodes, or failure of thriving, was considered to be inappropriate.

Early development of T cells obtained during PEG-ADA treatment was crucial to the implementation of the gene therapy protocol. Administration of PEG-ADA continued throughout the study period, although at decreasing amounts. Therefore, the relative role of gene-corrected cells and PEG-ADA treatment remains to be completely defined, an issue that will be addressed during the continuation of this study.

The aim of our study was to evaluate the safety and efficacy of the retroviral vector–mediated gene transfer procedure and to define the relative role of PBLs and BM stem and progenitor cells as effectors of long-term reconstitution of immune functions after gene transfer. For this purpose, we constructed two different retroviral vectors, DCA*l* and DCA*m*, expressing the human ADA cDNA under the control of its own promoter, which were used to infect PBLs and BM cells, respectively (Fig. 1). Both vectors are based on the double-copy (DC) design (32) and are structurally identical except for the presence of alternative restriction sites (Mlu I in DCA*l* and Bss HII in DCA*m*) in a nonfunctional region of the viral LTR (33). This feature allowed unequivocal tracing of the origin (BM or PBL) of the transduced cell progeny in the circulation by a simple polymerase chain reaction (PCR) analysis on genomic DNA (34). Both vectors were packaged in the amphotropic GP+*env* Am12 cell line (33). PBLs and T cell–depleted BM cells were transduced ex vivo either by multiple exposure to cell-free viral supernatant or by coculture with irradiated packaging cells (35, 36). Gene transfer efficiency increased from 1 to 2.5% up to 40% in total PBLs, with the introduction in the procedure of a new packaging line and of cocultivation. Gene transfer efficiency into CFU-GM and BFU-E hematopoietic progenitors averaged 30 to 40%, as described (37). These frequencies were estimated by cloning in lim-

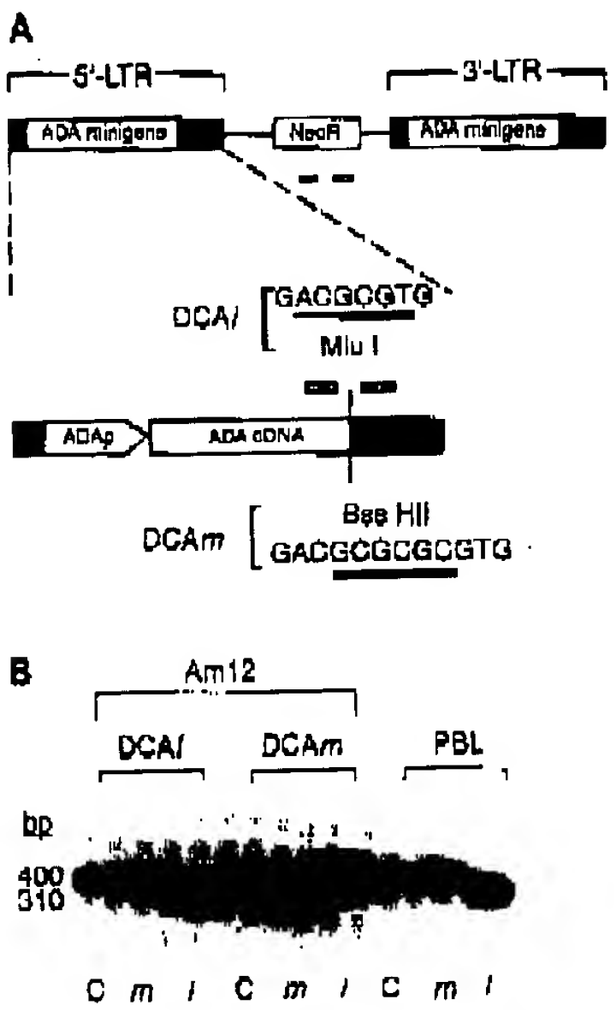

Fig. 1. (**A**) Structure of the DCA*l* (lymphocytes) and DCA*m* (marrow) proviruses. A human ADA minigene (promoter + full-length cDNA) was inserted into the LTR U3 region of a Moloney murine leukemia virus–derived retroviral vector (DCA) (32, 33). For construction of two vectors that could be distinguished from each other after integration into the target cell genome, the unique Mlu I restriction site present in a functionally irrelevant region of the LTR in DCA*l* was converted into a Bss HII site in DCA*m* (enlarged map). The hatched boxes indicate the location of the PCR primers used to detect vector DNA in target cells and for vector identification. (**B**) PCR identification of the vector integrated into the lymphocytes of patient 1 3 months after initial administration of DCA*l*-transduced PBLs and DCA*m*-transduced BM cells, showing the PBL origin of the transduced circulating lymphocytes. C, control (uncut PCR product); *m*, marrow-specific Bss HII cut present in DCA*m*-transduced cells; *l*, lymphocyte-specific Mlu I cut present in DCA*l*-transduced cells. PCR amplification of DNA obtained from the DCA*l* and DCA*m* packaging cell lines (Am12) is shown as a control.

iring dilution (38) and semi-solid colony-forming assays (39), respectively, in the presence or absence of G418 and are the result of the steady improvement in both cell-free infection and cocultivation that we have produced in recent years (10, 11, 18, 37). In particular, our goal has been to increase gene transfer frequency while maintaining phenotype, immune repertoire, and in vivo potential for proliferation, differentiation, and survival. For this purpose, short cultivation time under conditions of low interleukin-2 (IL-2) concentration were developed for the activation and infection of PBLs (35), while BM cells were maintained in a long-term culture system over adherent layers without addition of exogenous growth factors, and were infected during the first 3 days of culture (36). This system produces minimal loss of differentiation capacity and potential for in vivo hematopoietic reconstitution (40). No G418 selection was applied to infected PBLs or BM cells before reinfusion. Transduction efficiency and production of the vector-derived ADA in infected cells was determined by PCR and thin-layer chromatography (TLC), respectively (41).

In vivo administration of genetically modified cells began in March 1992 for patient 1 and July 1993 for patient 2. Patient 1 received 7.24×10^8 DCAl-transduced lymphocytes and 0.35×10^8 DCAm-transduced progenitor cells in nine injections administered intravenously (i.v.) over a period of 24 months. Patient 2 received a slightly smaller number of cells in five injections over 10 months.

We began monitoring the persistence of vector-transduced cells at monthly (or bimonthly) intervals from the first infusion. Analyses were performed both on bulk populations of cells of different origin, for the indication of origin of transduced cells, and on clonal assays for quantitation of transduced BM and PBLs. Six months after the beginning of treatment, long-term survival of transduced cells was demonstrated in both patients by the presence of vector-derived sequences in the DNA extracted from peripheral blood mononuclear cells, total BM cells, mature granulocytes (Fig. 2A), individual T lymphocyte clones (Fig. 3), and BM progenitors (BFU-E, CFU-GM, and CFU-GEMM) in clonal culture (41). ADA production at levels substantially greater than observed in untransduced ADA controls was observed in PBLs, BM, and granulocytes (Fig. 2C). The proportion of genetically modified cells in BM and circulating blood was monitored throughout the study by BM and T cell clonal assay in the presence of G418, and indirectly from the amount of ADA activity in total cell populations from BM and peripheral blood. In both patients, this proportion ranged between 5 and 30% of clonable BM progenitors and between 0.8 and 8.5% in PBLs. Total ADA activity ranged between 5 and 18% of normal values in both patients' nucleated cells in the blood. Sixteen months after discontinuation of treatment, ADA activity in total circulating nucleated cells was 126 nmol hour^{-1} per milligram of protein in patient 1 and 77 nmol hour^{-1} per milligram of protein in patient 2 (internal normal control, 1157 nmol hour^{-1} per milligram of protein; for method see legend to Fig. 2). At the same time, in patient 1, the frequency of transduced G418-resistant T cell was 4.76% and that of clonable BM progenitors was 25%; in patient 2 these frequencies were 2.01 and 17%, respectively. During this period in patient 1, and more recently in patient 2, ADA activity became reproducibly detectable also in circulating erythrocytes (Fig. 2C). Vector-derived ADA activity in individual T cell clones was comparable to, or higher than, that of normal controls (legend to Fig. 2C), as observed in T cells that survived in vivo selection in the human PBL-SCID mouse preclinical model (10, 11). ADA activity in Neo-resistant BM colonies also averaged normal levels (41).

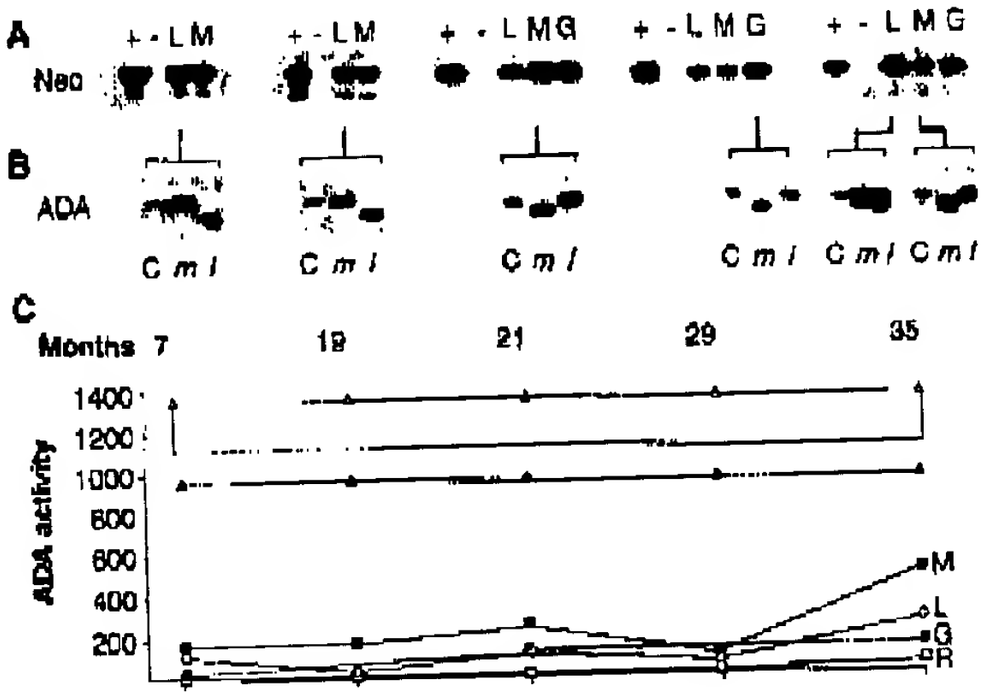

Fig. 2. Persistence of transduced hematopoietic cells in vivo and analysis of their origin. During 3 years after initiation of the gene therapy trial, persistence of transduced PBLs and BM cells, and expression of vector-derived ADA activity were documented at regular intervals. (**A**) Detection of transduced cells by PCR analysis for the NeoR gene was consistent throughout the follow-up of patient 1 in PBLs (L), BM cells (M), and circulating granulocytes (G). + and −, PCR positive and negative controls, respectively. (**B**) Analysis of the identity of the integrated vector showed that vector-positive lymphocytes were initially all derived from long-lived transduced PBLs, as demonstrated by the presence of the DCA*l*-specific PCR pattern (7 and 19 months), whereas BM and granulocytes showed the DCA*m*-specific pattern (21, 29, and 35 months). Three years after initiation and 1 year after discontinuation of treatment, DCA*m*-specific signals started to appear in the DNA extracted from PBLs, indicating progressive conversion of the circulating, genetically modified lymphocyte pool from a predominantly PBL-derived to a BM progenitor–derived population (35 months). This observation was further confirmed by the analysis of Neo-resistant, peripheral blood T cell clones (Fig. 3). (**C**) In parallel, vector-derived ADA activity was monitored by TLC in total PBLs (L) and BM cells (M) of patient 1. G, granulocytes; R, red blood cells. Two positive controls are provided: ADA activity (mean ± SE) from a pool of normal individuals (△), and ADA activity from a polyclonal PBL line from the same patient transduced in vitro and selected in G418 (▲). Lysates were prepared from 1×10^6 cells in 10 μl of CGLB buffer by three cycles of freeze and thaw. ADA enzyme activity was analyzed by the ^{14}C-adenosine to ^{14}C-inosine conversion assay followed by TLC (37). Cell lysates from individual clones (~2×10^5 cells) were normalized for protein content by the BIO-RAD protein assay (Bio-Rad Laboratories GmbH, Munich, Germany). Positive and negative controls were, respectively, lysates from normal PBLs and uninfected, IL-2–stimulated ADA$^+$ PBLs, because IL-2 stimulation is reported to increase the efficiency of ADA expression in ADA$^+$ cells (37). TLC plates were exposed for 3 days in a PhosphorImager (Molecular Dynamics, Sunnyvale, California). Ratio of adenosine conversion is expressed as nmol hour^{-1} mg^{-1}.

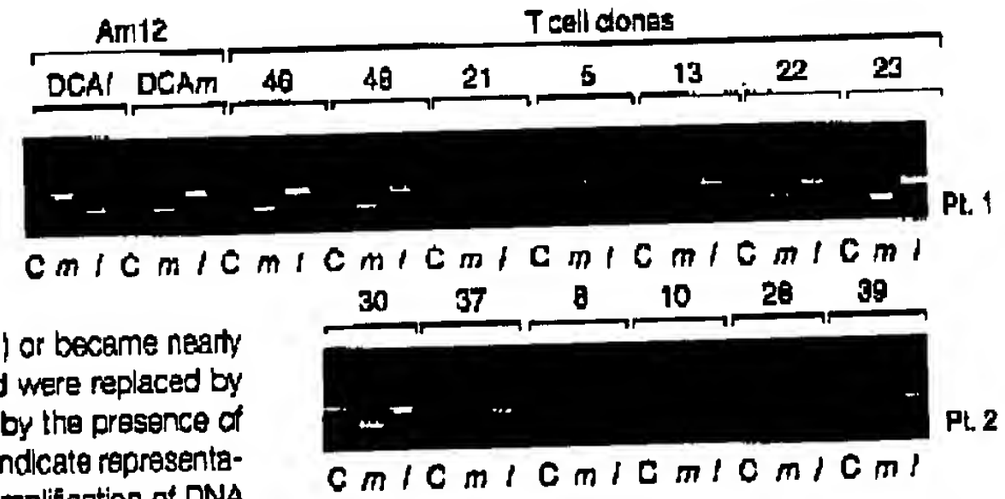

Fig. 3. Origin of T cell clones obtained from the peripheral blood of patient 1 (top) and patient 2 (bottom) 1 year after discontinuation of treatment. Clonable T cells containing the DCA*l* vector diminished markedly (patient 1) or became nearly undetectable (patient 2), and were replaced by BM-derived T cells, marked by the presence of the DCA*m* vector. Numbers indicate representative individual clones. PCR amplification of DNA obtained from the DCA*l* and DCA*m* packaging cell lines (Am12) is shown as a control.

Spontaneous revertants (ADA-positive, vector-negative) were not observed in either peripheral blood or BM.

Initially, the analysis of the retroviral vector amplified from the DNA of circulating lymphocytes indicated that genetically modified cells were derived from a pool of long-lived PBLs originally transduced with the DCAI vector (Fig. 1B). This finding was consistent throughout the period of administration of transduced PBLs and BM cells (Fig. 2B, at 7 and 19 months, for example), whereas total BM cells (Fig. 2B, at 21 and 35 months, for example) and circulating granulocytes (Fig. 2B, at 29 months, for example) always showed the DCAm-specific restriction pattern of marrow-derived cells. However, about 1 year after discontinuation of gene therapy, both PBL- and BM-derived lymphocytes were detectable in the circulation (Fig. 2B, at 35 months). At that time, Neo-resistant, clonable T cells containing the PBL-specific DCAI vector sharply decreased (Fig. 3, patient 1) or became undetectable (Fig. 3, patient 2) and were progressively replaced in the circulation by T cells containing the BM-specific, DCAm vector. To confirm this important finding, we evaluated two additional time points, subsequent to the data in Figs. 2 and 3, on bulk populations and on T lymphocyte clones. Thirty-eight clones from patient 1 were analyzed for their origin; 26 were derived from marrow, 6 could not be unequivocally determined, and 9 contained the DCAI vector. Similarly, of 49 clones obtained from patient 2, 6 could not be clearly determined, 6 contained the DCAI vector, and all others were derived from marrow.

These results show that short-term immune reconstitution was sustained in the two patients by a population of peripheral blood–derived, ADA-producing lymphocytes with a life-span in the circulation ranging between 6 and 12 months. We have previously shown that this population contains both mature T cells and immature, or naive, precursors (*11*, *18*). Conversely, long-term reconstitution resulted almost exclusively from transduced, BM-derived hematopoietic stem and progenitor cells capable of generating multilineage progenies of ADA-producing cells, that is, lymphocytes, granulocytes, and (more recently) erythrocytes.

A fundamental hypothesis underlying this study was the possibility that genetically corrected cells would benefit from a selective advantage over noncorrected cells. Our experimental design has made it possible to obtain data in support of this hypothesis. The first line of evidence comes from the progressive appearance of marrow-derived PBLs, generated over time from a relatively small number of genetically modified precursors contained in the transduced marrow cell population (Figs. 2 and 3). Additional evidence comes from the analysis of the integrated retroviral vectors. In a recent comparative analysis of different vector constructs designed for gene transfer of reporter genes in human PBLs, we demonstrated that the DC construct carries an inherent instability that results in loss of the gene inserted in the viral LTR (*18*). Such instability could affect 50% of integrated proviruses, depending on the size and nature of the inserted gene. In the present study, the analysis of over 200 T cell clones obtained at different times during the follow-up of the two patients showed no rearrangement that might have eliminated the ADA gene, and consequently its expression. Conversely, loss of the ADA gene could be detected only in marrow-derived colonies and T cell clones that had been transduced and cultured in vitro, in the absence of any positive selection (*41*). These observations indicate that, in ADA$^-$ SCID patients, ADA-producing cells have a selective advantage over noncorrected ADA cells, as previously suggested in the

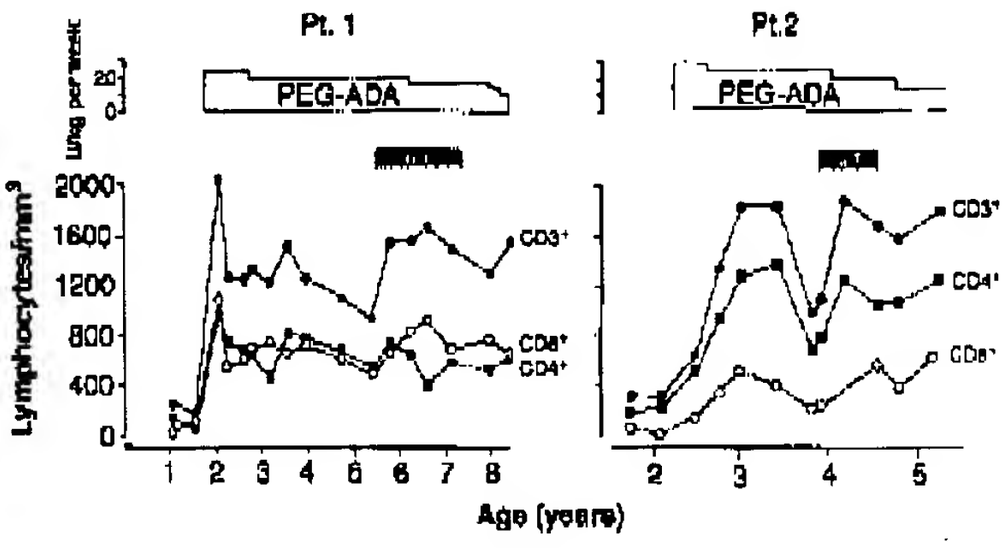

Fig. 4. Immune reconstitution during the PEG-ADA and gene therapy trial for patient 1 (left) and patient 2 (right). CD3$^+$, CD4$^+$, and CD8$^+$ lymphocyte counts are plotted against age for the duration of the trial. Doses of PEG-ADA administered to the patients are shown in the upper part of the graphs. The black boxes (GT) indicate the period of administration of genetically modified PBLs and BM cells (ticks indicate individual injections).

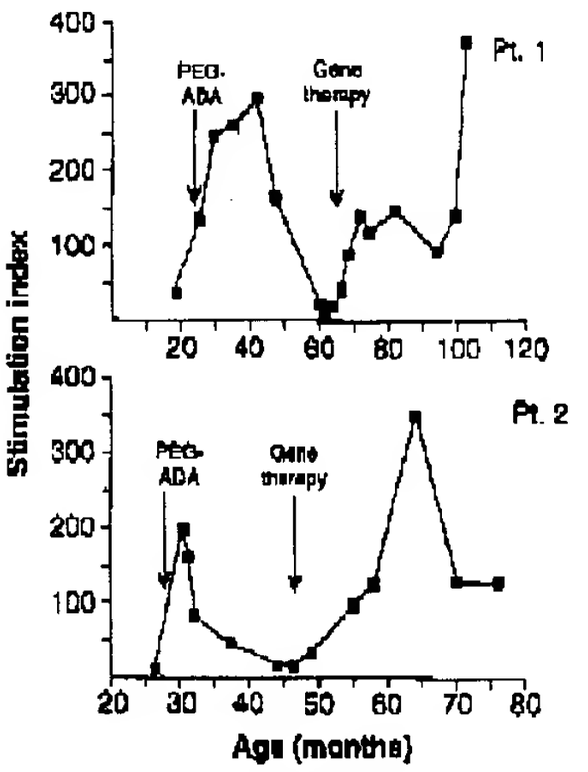

Fig. 5. Immune reconstitution during the PEG-ADA and gene therapy trial for patient 1 (top) and patient 2 (bottom). T cell proliferative response to mitogenic stimulus is presented as stimulation index (cpm of stimulated samples divided by cpm of unstimulated cells) and is plotted against age of the patients. Shaded areas indicate the range of the stimulation index of normal internal controls. Response to TT followed a comparable kinetics. Arrows indicate initiation of enzyme replacement therapy (PEG-ADA) and administration of genetically modified cells (gene therapy).

Table 1. Quantitation of isoagglutinin titer, and antigen-specific antibody production and proliferative response after vaccination with tetanus toxoid (TT). IgG, immunoglobulin G; ND, not done.

Time of test	Anti-B isoagglutinin titer		Serum titer of anti-TT IgG*		Proliferative response (10^3 cpm)†	
	Patient 1	Patient 2	Patient 1	Patient 2	Patient 1	Patient 2
Before immunization	ND	1/2	2.4	0.2	0.5	1.6
PEG-ADA response	1/8	1/16	1600	130	26.4	15.7
PEG-ADA failure	1.8	1.5	ND	34	1.8	1.5
After gene therapy I	1/16	1/32	ND	88	50.3	67.6
After gene therapy II	1/32	1/32	ND	ND	82.2	96.5

*Anti-TT IgG production was determined in a standard enzyme-linked immunosorbent assay and is reported as international units to a reference standard (Biagini Reference Standard, Biagini, Florence, Italy). †TT-specific T cell lines were tested for their capacity to proliferate in response to TT in the presence of autologous antigen-presenting cells (*45*). Proliferation in response to antigen-presenting cells alone was always <1000 cpm. Both patients received the full immunization schedule with TT (three doses) while on PEG-ADA. Patient 2 showed signs of immune deterioration during the immunization schedule. Isoagglutinin titer, TT-specific IgG titer, and TT-specific T-cell proliferation were measured at the following times: before immunization and before use of PEG-ADA (2 years and 5 months of age for patient 1 and 2 years and 10 months for patient 2), at the time of peak response to PEG-ADA (2 years since the beginning of PEG-ADA treatment for patient 1 and 1 year for patient 2), at PEG-ADA failure, and twice after gene therapy (6 and 8 years of age for patient 1 and 4.5 years and 5 years for patient 2).

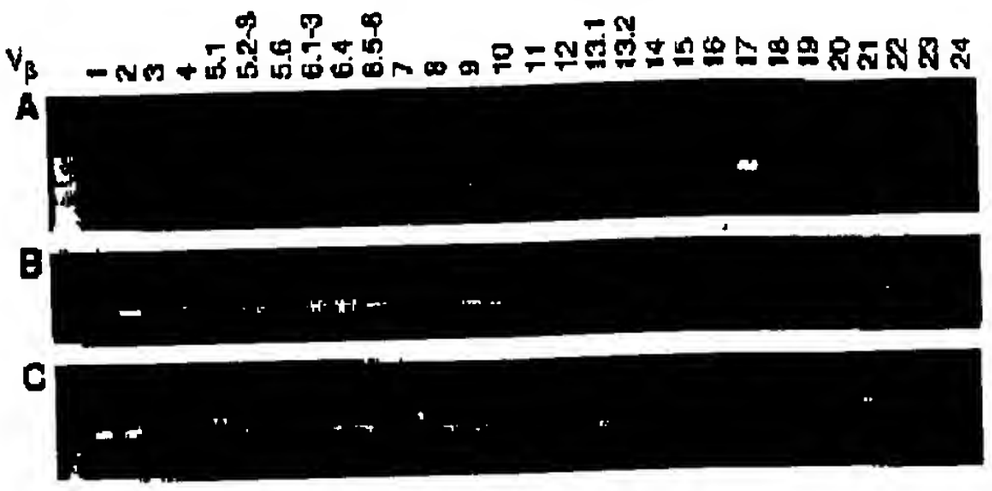

Fig. 6. Development of a normal T cell receptor repertoire in patient 1 after gene therapy treatment. Different T cell receptor V_β-chain usage at the time of failure of the PEG-ADA treatment (**A**), 1 year after the beginning of gene therapy (**B**), and 1 year after discontinuation of transduced cell administration (**C**), as analyzed by RT-PCR amplification with V_β chain-specific primers (48).

human PBL–SCID mouse preclinical model (10, 11).

Immune reconstitution induced by PEG-ADA treatment lasted for over 3 years in patient 1 and for a shorter period in patient 2, despite administration of a 50% higher PEG-ADA dose in the latter (Figs. 4 and 5). In association with a progressive decline in PBL counts, the immune response decreased markedly over a short period of time (Figs. 4 and 5 and Table 1). Administration of genetically modified cells rapidly restored immune functions in both patients, resulting in normalization of total lymphocyte counts (Fig. 4) and cellular and humoral responses, including sustained isohemagglutinin titer, antigen-specific antibody production, and mitogen- and antigen-specific proliferation (Fig. 5 and Table 1). The T cell receptor repertoire, analyzed by the V_β chain usage, normalized progressively (Fig. 6). In patient 2, the overall response to gene therapy was similar to that of patient 1, despite administration of a smaller number of genetically modified PBLs and BM cells. There have been no serious infections in the two patients throughout the PEG-ADA treatment and after the beginning of gene therapy. The patients received no other treatment, except for high-dose immunoglobulins administered intravenously and prophylactic antibiotic treatment, both of which were discontinued after indications of full immunologic reconstitution. Before the beginning of the PEG-ADA treatment, the patients showed severe growth failure, ranging below the fifth percentile for height and weight. Enzyme replacement and gene therapy had a marked clinical impact, resulting in normalization of height and weight. Patient 2, who had a very limited initial response to PEG-ADA, resumed normal growth only after gene therapy. Serum chemistry values, blood counts, and urinalysis indicated no toxicity from PEG-ADA or gene therapy treatments. Monitoring of the two patients for the presence of recombinant helper virus was consistently negative.

The results of the long-term follow-up have two main implications: the selection of an optimal treatment for ADA SCID patients and, more generally, the potential application of similar gene therapy approaches to the treatment of genetic and acquired diseases. Our study clearly indicates the feasibility of direct BM cell gene therapy; however, in specific circumstances, genetically modified PBLs may provide a prompt supply of immune effector cells until development of BM-derived lymphocytes. If it is proven to be efficacious over time, this procedure could represent a less toxic alternative to unrelated or HLA-mismatched marrow transplants. In the prospective extension of these results to the design of other gene therapy clinical trials, gene transfer into hematopoietic progenitors can be achieved also in the absence of the stress conditions associated with cytoreduction and BM transplantation. However, in steady-state hemaropoiesis a considerable time lag may be required before appearance of genetically modified cells in the blood. Under these conditions, the positive selection may represent an absolute requirement for favoring the appearance of vector-transduced cells. Such positive selection may be "naturally" present in other genetic or acquired diseases [for example, acquired immunodeficiency syndrome (AIDS)] or could be built into the vector as a drug-resistance gene.

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31. Both patients were vaccinated with tetanus toxoid (TT; Swiss Serum Institute, Bern, Switzerland). To evaluate the number of precursor lymphocytes specific to TT, the frequency of cells capable of proliferating in the presence of irradiated autologous PBLs that had been pulsed overnight with TT was evaluated in a limiting dilution assay. At two different times after vaccination, the frequency of TT-specific lymphocyte precursors was 1:2500 and 1:5000 in patient 1. This range is comparable to that of normal individuals at the same time after immunization. Patient 2 showed signs of immune deterioration during the immunization schedule (Table 1).
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33. The double-copy DCA retroviral vector, carrying a human ADA minigene, has been described previously (*32*) and is indicated as DCA/ in this report. The DCAm vector was obtained by digestion, Klenow-filling, and religation of the DCA/ plasmid at the Mlu I restriction site in the polylinker region of the 3′ LTR, thus generating a new Bss HII site. The Am12/DCAm-7 and the Am12/DCA/-18 clonal packaging cell lines were generated by the transinfection protocol, as described (*18*).
34. High molecular weight DNA was obtained from cells by standard phenol-chloroform extraction (*42*). A Neo-specific, 382-bp fragment located in the coding region of the NeoR gene or a DCA-specific 400-bp fragment spanning the 3′ end of the ADA cDNA, the polylinker, and part of the LTR U3 region, were amplified from 0.5 μg of genomic DNA by 30 cycles of PCR with 5 U of Taq polymerase (Perkin Elmer, Norwalk, CT) and 25 pmol of the primers Neo-1 (5′-GGAAGCCGGTCTTGTCGATC-3′), Neo-3 (5′-AGAGTCCCGCTCAGAAGAAC-3′), DCA-5 (5′-TCAATGCGGCCAAATCTAG-3′), and DCA-6 (5′-GCTGTTCCATCTGTTCCTGA-3′). DCA-specific PCR products were digested by Bss HII and Mlu I restriction enzymes (Boehringer Mannheim GmbH, Mannheim, Germany). Reaction mixtures (1/10th of the total volume) were separated on a 1.5% agarose gel, and DNA was visualized by ethidium bromide staining. DNA was transferred to a nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK) by DNA capillary blotting (*42*) and hybridized to 10^7 dpm of ^{32}P-labeled, 1.2-kb Hind III–Sma I fragment of pSV2-neo (*43*), or 1.9 Kb Xho I fragment of the ADA cDNA. Filters were washed under high-stringency conditions and exposed to Kodak X-AR5 film for 30 min to overnight at −70°C.
35. Freshly isolated ADA PBLs were obtained from ADA SCID patients, Ficoll-fractionated, and grown at 10^6 cells/ml in 24-well tissue culture plates under phytohemagglutinin (PHA) (2 μg/ml; Boehringer Mannheim GmbH) and human recombinant IL-2 (hu-rIL-2, 100 U/ml; Eurocetus B.V., Amsterdam, Netherlands) stimulation in lipopolysaccharide-free RPMI 1640 medium supplemented with 2 mM L-glutamine and 5% human serum. In subsequent experiments similar levels of gene transfer could be obtained at 50 or 100 U of hu-rIL-2 per milliliter, in the absence of any additional stimulus (C. Benati *et al.*, in preparation). After 72 to 96 hours of stimulation, T lymphocytes were cocultivated with irradiated (10,000 roentgen) vector-producing cells for 72 hours in complete Dul-

Dulbecco's minimum essential medium (DMEM) in the presence of polybrene (8 μg/ml) as described (18). Cells were washed twice in phosphate-buffered saline (PBS), resuspended in fresh medium, and cultured for 3 to 4 days. Transduced cells were tested for the presence of helper virus and cryopreserved until use.
36. BM mononuclear cells were obtained as a Ficoll fraction and grown for 2 to 3 days in complete DMEM at a density of 6×10^5 to 8×10^5 cells/cm^2 (35). T cell depletion and progenitor cell enrichment were obtained as described (3, 35). Gene transfer was carried out by multiple infection cycles with cell-free, helper virus–tested viral supernatants in the presence of polybrene (8 μg/ml) (36). BM cells were maintained in a long term culture system over adherent layers without addition of exogenous growth factors, and infected during the first 3 days of culture. Transduced cells were tested for the presence of helper virus and cryopreserved until use. At that time, the transduced cells were washed, resuspended in normal saline containing 4% human albumin, and reinfused into the patient.
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38. PHA blasts or antigen-specific T cells were cloned by limiting dilution. The relative frequencies of transduced cells was obtained by comparing the precursor frequency in the absence and presence of G418 (800 μg/ml). G418-resistant T cell clones were isolated and maintained as described (*44*, *45*).
39. The relative frequencies of transduced BM progenitor cells were obtained by comparing the frequency of CFU-G, CFU-GM, BFU-E, and CFU-GEMM cells in the absence and presence of increasing doses of G418 (0.7, 1.0, 1.5 mg/ml) as described (37). In selected experiments, individual G418-resistant colonies were collected for analysis of vector transduction and expression.
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48. T cell receptor V_β-chain usage was analyzed on transduced T cell lines by reverse transcriptase–PCR. Briefly, total RNA was reverse transcribed with oligo(dT) and oligo(dG) primers and subjected to PCR with V_β- or C_μ-specific oligonucleotides (*46*) or to anchored PCR with a C_β-specific oligonucleotide as described (*47*). Amplified products were analyzed by agarose gel electrophoresis.
49. We are indebted to L. Ruggieri and A. Wack for performing some of the ex vivo and in vitro analyses of gene transfer frequency; to the nurses and clinical staff of the Clinica Pediatrica, School of Medicine, University of Brescia, for skilled and dedicated care; to A. Amghini and A. Crescenzo for clinical assistance in the extended care of the two patients; to A. Plebani for dosing specific antibody production; to M. Hershfield, P. Deltabona, and A. Ballabio for helpful discussions; and to Enzon, Inc., and Ophan Europe for providing PEG-ADA before commercial distribution. Supported by grants from Telethon, the Italian National Research Council, and the Italian Ministry of Health (IV-VII AIDS Projects).

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T Lymphocyte–Directed Gene Therapy for ADA$^-$ SCID: Initial Trial Results After 4 Years

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In 1990, a clinical trial was started using retroviral-mediated transfer of the adenosine deaminase (ADA) gene into the T cells of two children with severe combined immunodeficiency (ADA SCID). The number of blood T cells normalized as did many cellular and humoral immune responses. Gene treatment ended after 2 years, but integrated vector and ADA gene expression in T cells persisted. Although many components remain to be perfected, it is concluded here that gene therapy can be a safe and effective addition to treatment for some patients with this severe immunodeficiency disease.

The possibility of using gene transfer as a therapy for human disease has great appeal. The decision to enter clinical trials awaited the development of safe and efficient techniques of gene transfer and improved understanding of the basic pathology and biology underlying likely candidate diseases and target cells. The advent of useful retroviral vectors that permitted relatively high efficiency gene transfer and stable integration was a critical advance (*1*, *2*), as was the demonstration that this procedure of gene transfer could be effectively and safely used in humans (*3*).

Severe combined immunodeficiency secondary to a genetic defect in the purine catabolic enzyme adenosine deaminase [ADA SCID] is characterized by defective T and B cell function and recurrent infections, often involving opportunistic pathogens. Large amounts of deoxyadenosine, an ADA substrate, are present in these patients; deoxyadenosine is preferentially converted to the toxic compound deoxyadenosine triphosphate in T cells, disabling the immune system (*4*).

Because this disease is curable by allogeneic bone marrow transplantation given without pretransplantation cytoreductive conditioning, it was initially assumed that gene therapy should be directed at the bone marrow stem cell. However, initial attempts to use stem cell gene transfer in primates resulted in only low-level, transient gene expression, insufficient for clinical use. The observation that the only donor cells detected in some patients "cured" by allogeneic bone marrow transplantation was their T cells—the others remaining ADA-deficient (*5*)—raised the possibility that T cell–directed gene therapy also might be a useful treatment.

The introduction of enzyme replacement with ADA-containing erythrocytes (*6*) or with bovine ADA conjugated with polyethylene glycol (PEG-ADA) (*7*) has made this approach feasible. PEG-ADA has provided noncurative, life-saving treatment for ADA SCID patients; with this treatment, most patients have experienced weight gain and decreased opportunistic infections. Full immune reconstitution has been less regularly achieved with enzyme therapy. T cell function as measured by in vitro mitogen responses improved in most patients, but fewer patients recovered consistent immune responses to specific antigens [for instance, as measured by normal delayed-type hypersensitivity (DTH) skin test reactivity] (*8*–*10*). Nearly all PEG-ADA–treated patients showed increased peripheral T cell counts, which provided a source of T cells for gene correction not available without enzyme therapy. Furthermore, enzyme treatment could be continued during the gene therapy trial so that the ethical dilemma of withholding or stopping a life-saving therapy to test an unknown treatment could be avoided.

The adenosine deaminase complementary DNA (cDNA) (*11*) is 1.5 kb and fits within a retroviral vector. With the use of an ADA-containing retroviral vector, ADA-deficient T cell lines were transduced to express normal amounts of ADA; this rendered them normally resistant to intoxication and growth inhibition when challenged with deoxyadenosine (*12*, *13*). Next, studies in mice, rabbits, and nonhuman primates using T cells modified with retroviral vectors showed normal cell survival and function after their reintroduction into recipient animals (*14*). Finally, Bordignon and colleagues (*15*) showed that ADA gene–corrected T cells acquired a survival advantage compared with uncorrected ADA-deficient cells when transplanted into immunodeficient, but ADA-

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